Supporting Information

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SI Materials and Methods

Plasmids and Reagents. NOD2FL, NOD2ΔCARD1, and NOD2ΔCARDs were cloned into p3XFLAG-CMV-14 (Sigma). NOD2ΔLRR, NOD2CARDs, caspase-1CARD, caspase-1ΔCARD, and NOD2FL fused to different epitope tags (FLAG, c-Myc, or HA), were cloned into pcDNA3 (Invitrogen). Full-length NOD2 was cloned into the retroviral vector pMSCVpuro (Clontech). Recombinant LF and PA were obtained as described and combined to generate LT (1). LPD (*Eschericia coli*, 055:B5) and MDP were purchased from Sigma-Aldrich and Bachem, respectively.

Cytokine Secretion and Cell Signaling. Whole-cell extracts were separated by SDS/PAGE, transferred to Immobilon membranes (Millipore), and analyzed by immunoblotting. Cytokines were quantitated by ELISA by using duoSet kits from R&D Systems. Il-1β (Cell Signaling), and caspase-1 (2).

Transfections and Retroviral Infection. HEK293T cells were transfected by using Lipofectamine 2000 (Invitrogen) in the absence or presence of MDP. Cell lysates were analyzed by immunoprecipitation and immunoblotting as described (3). Bone marrow-derived macrophages (BMDMs) were retrovirally transduced as described (4). Culture supernatants were analyzed for IL-1β release and caspase-1 content.

shRNA-Medited Gene Silencing. Oligonucleotides (sequence available upon request) corresponding to NALP1-specific shRNA were cloned into pLSLPw (lentivirus vector) and lentiviruses were prepared. TDM were infected with lentivirus as described (1) and cultured for 72 hr before incubation with 10 μg/ml MDP for 12 hr.

Size-Exclusion Chromatography. TDM were treated with 10 μg/ml MDP for 2 hr or left untreated. Cell pellets were resuspended in lysis buffer W (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF) supplemented with protease inhibitors and sonicated three times at 10-sec pulses with 30-sec intervals. Approximately 5 mg of protein lysate was loaded onto a precalibrated Superdex 200 column (Amersham-Pharmacia Biotech) and eluted in buffer W.

Analysis of Gene Expression and NF-κB Activity Assay. Total cellular RNA was prepared using TRIzol (Invitrogen), quantitated by UV absorption, and analyzed by real-time Q-PCR (3). Primer sequences are available upon request. All values were normalized to cyclophilin mRNA levels. NF-κB DNA binding was determined as described (5).

1. Park JM, *et al.* (2005) Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis–CREB and NF-kappaB as key regulators. *Immunity* 23:319–329.
2. Mariathasan S, *et al.* (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213–218.
3 Hsu LC, *et al.* (2004) The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. *Nature* 428:341–345.
4. Kagan JC, Roy CR (2002) Legionella phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat Cell Biol* 4:945–954.
5. Lawrence T, Bebien M, Liu GY, Nizet V, Karin M (205) IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation. *Nature* 434:1138–1143.

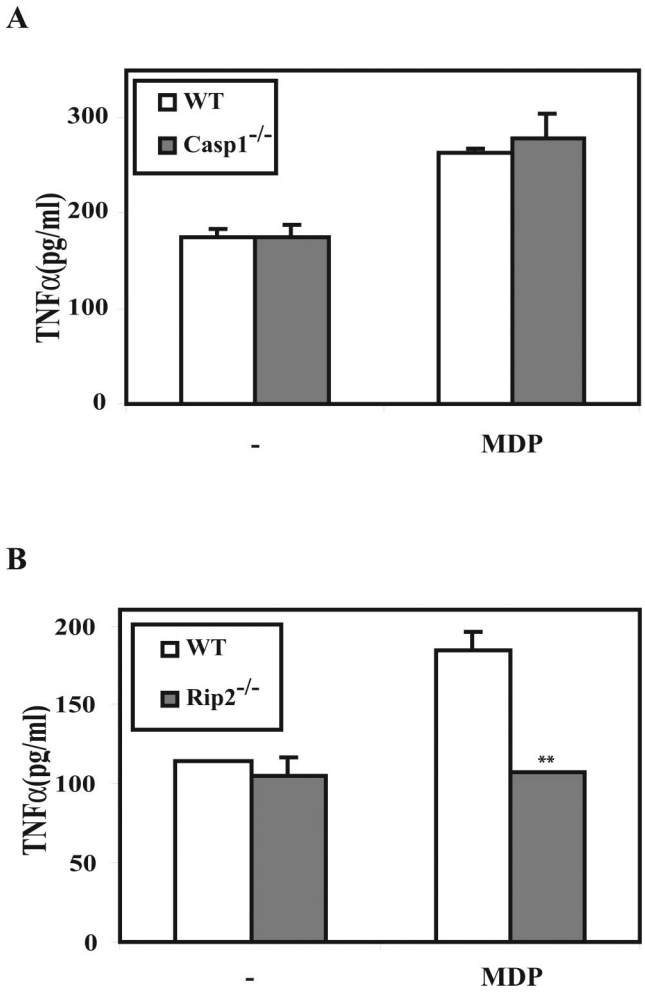

Fig. S1. RIP2 but not caspase-1 is required for elevated MDP-stimulated TNF-α secretion in macrophages. (*A* and *B*) Peritoneal macrophages from the indicated mouse strains were incubated with TiO_2 microparticles without or with MDP and TNF-α secretion was assayed by ELISA 16 h later. Results are averages of three separate experiments. Significant differences, $^{**}P < 0.01$, $^{*}P < 0.05$

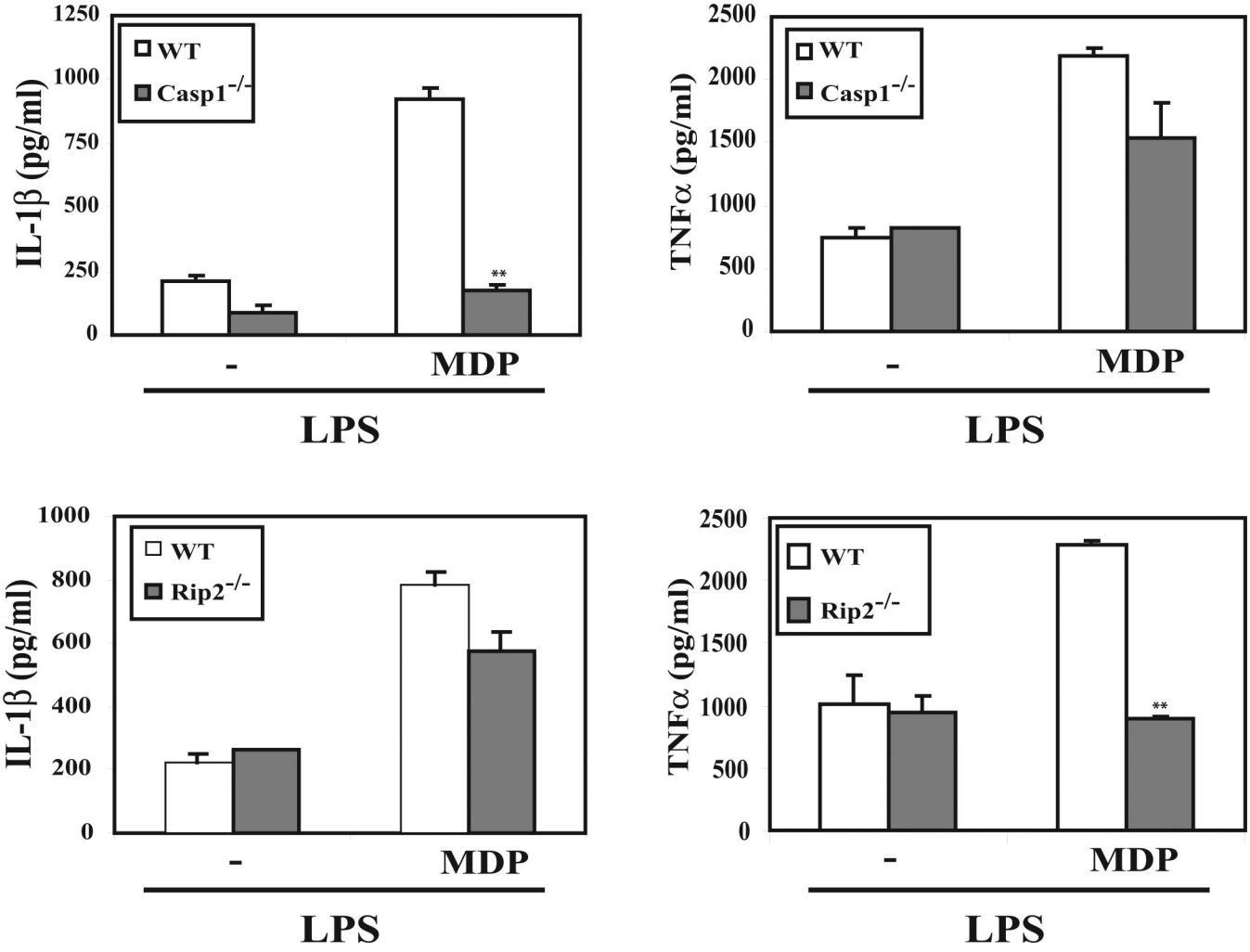

Fig. S2. RIP2 is required for TNF-α but not IL-1β secretion, whereas caspase-1 is essential for IL-1β but not TNF-α release in LPS-primed macrophages stimulated with MDP. LPS-primed peritoneal macrophages from the indicated mouse strains were incubated with TiO_2 microparticles without or with MDP. Secreted IL-1β and TNF-α were measured after 16 h by ELISA. Significant differences, $^{**}P < 0.01$, $^{*}P < 0.05$

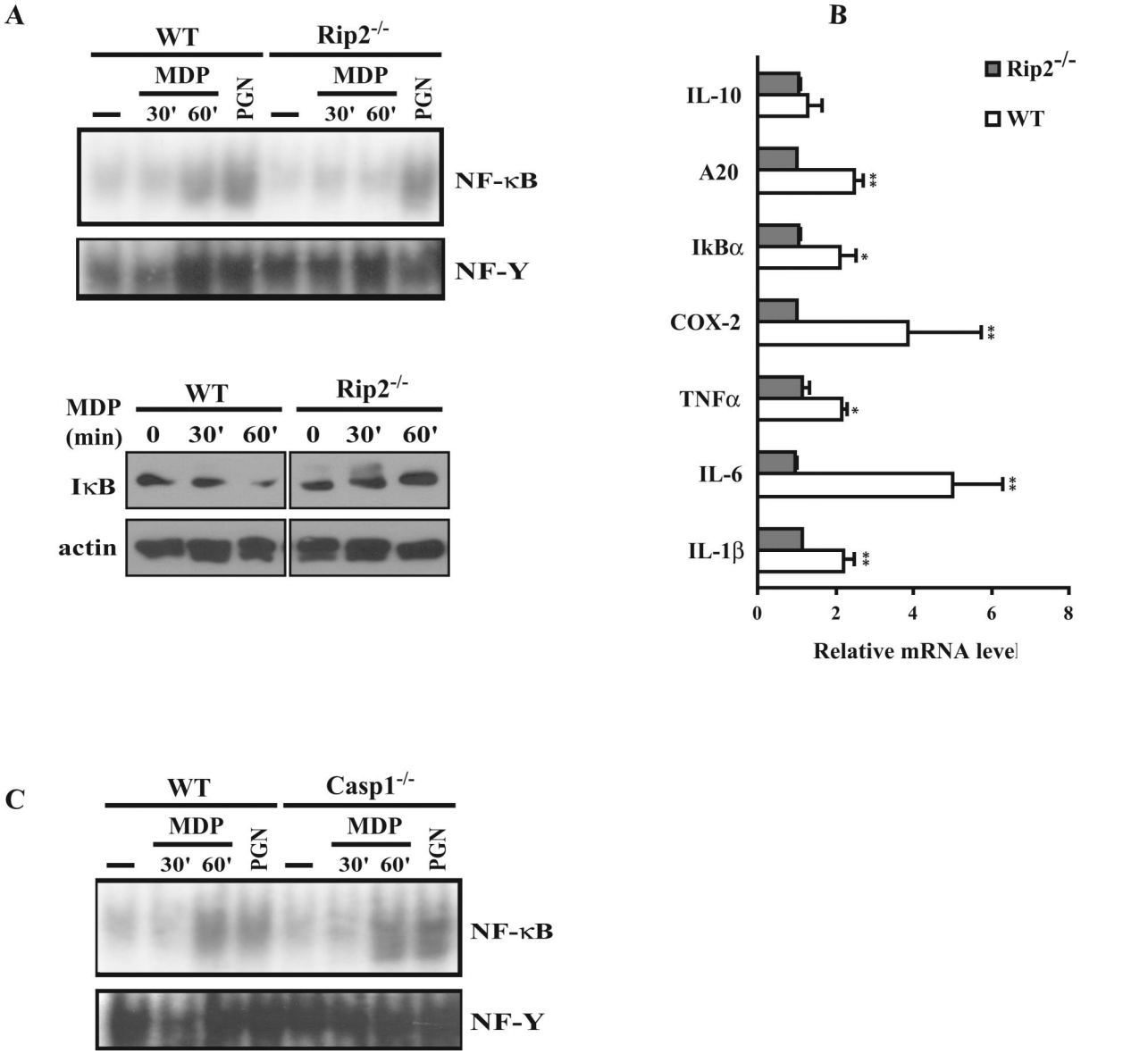

Fig. S3. NF-κB activation by MDP in macrophages requires RIP2 but not caspase-1. (*A*) RIP2 is required for MDP-dependent IκB degradation in macrophages. Peritoneal macrophages from the indicated mice were incubated for 30 or 60 min with TiO_2 particles without or with MDP (1 μg/ml) or peptidoglycan (PGN from *Staphylococcus aureus*, 10 μg/ml) for 30 min. Cell lysates were prepared and NF-κB DNA binding activity was analyzed by EMSA and IκBα degradation was examined by immunoblot analysis. Actin and NF-Y were used as loading controls. (*B*) RIP2 is required for induction of NF-κB target genes after MDP stimulation. Peritoneal macrophages were incubated with MDP with TiO_2 for 4 h. Total RNA was prepared and expression of the indicated genes was examined by real time Q-PCR. Data are presented as fold-increase in mRNA expression relative to non-stimulated WT macrophages, which were assigned an arbitrary level of 1.0 for each gene. Significant differences, $^{**}P < 0.01$, $^{*}P < 0.05$. (*C*) Caspase-1 is not required for MDP-induced NF-κB activation. Peritoneal macrophages from the indicated mouse strains were treated as above. NF-κB DNA binding activity was measured by EMSA. NF-Y activity was used as a loading control.

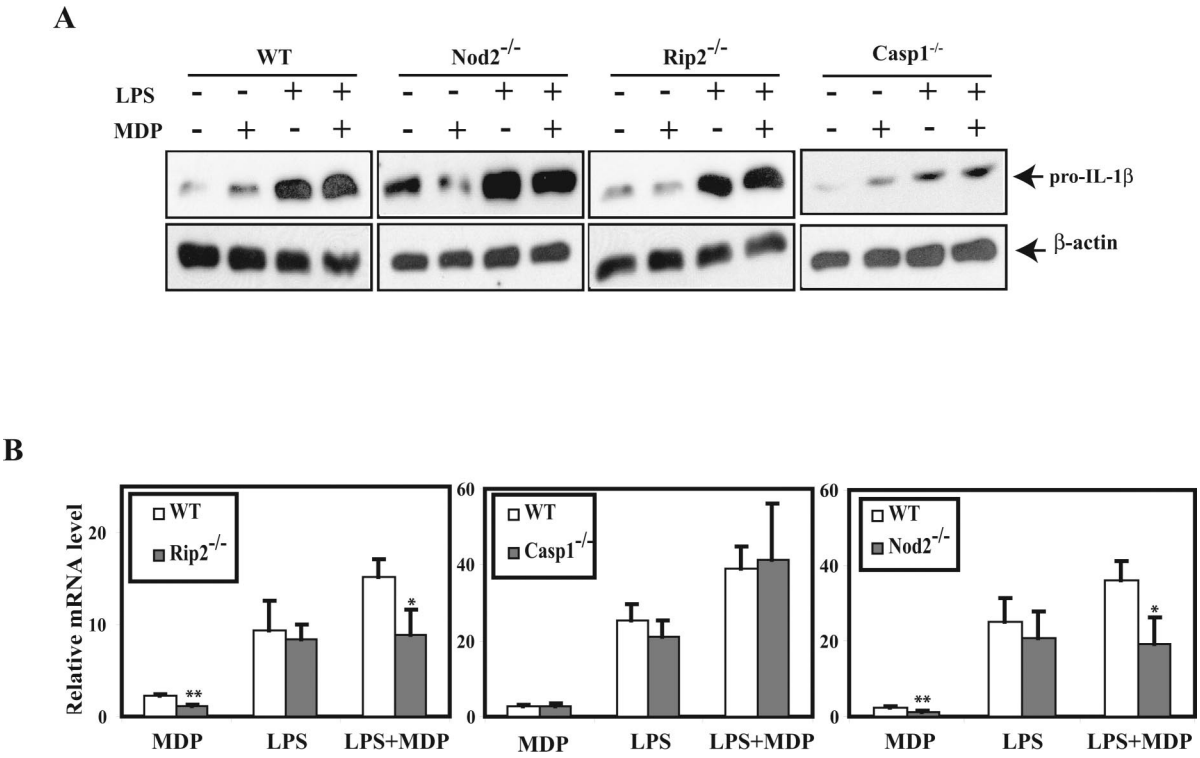

Fig. S4. Roles of NOD2, RIP2 and caspase-1 in induction of pro-IL-1β synthesis. (*A*) Peritoneal macrophages from the indicated strains were incubated without or with LPS (0.5 ng/ml) for 6 h, and then stimulated with TiO_2 microparticles without or with MDP (10 μg/ml) for 4 h. Cell lysates were prepared and pro-IL-1β was examined by immunoblot analysis. (*B*) NOD2, caspase-1 and RIP2 dependence of IL-1β mRNA induction. Peritoneal macrophages from the indicated strains were preincubated with or without LPS (0.5 ng/ml) for 6 h followed by 4 h incubation with TiO_2 microparticles without or with MDP. Total cellular RNA was isolated and pro-IL-1β mRNA levels were determined by real time Q-PCR. Data are fold-increase in mRNA expression in stimulated macrophages relative to non-stimulated macrophages, which were given an arbitrary level of 1.0. Results are means $\pm$ s.d. of three independent experiments normalized to the level of cyclophilin mRNA. Significant differences, $^{**}P < 0.01$, $^{*}P < 0.05$

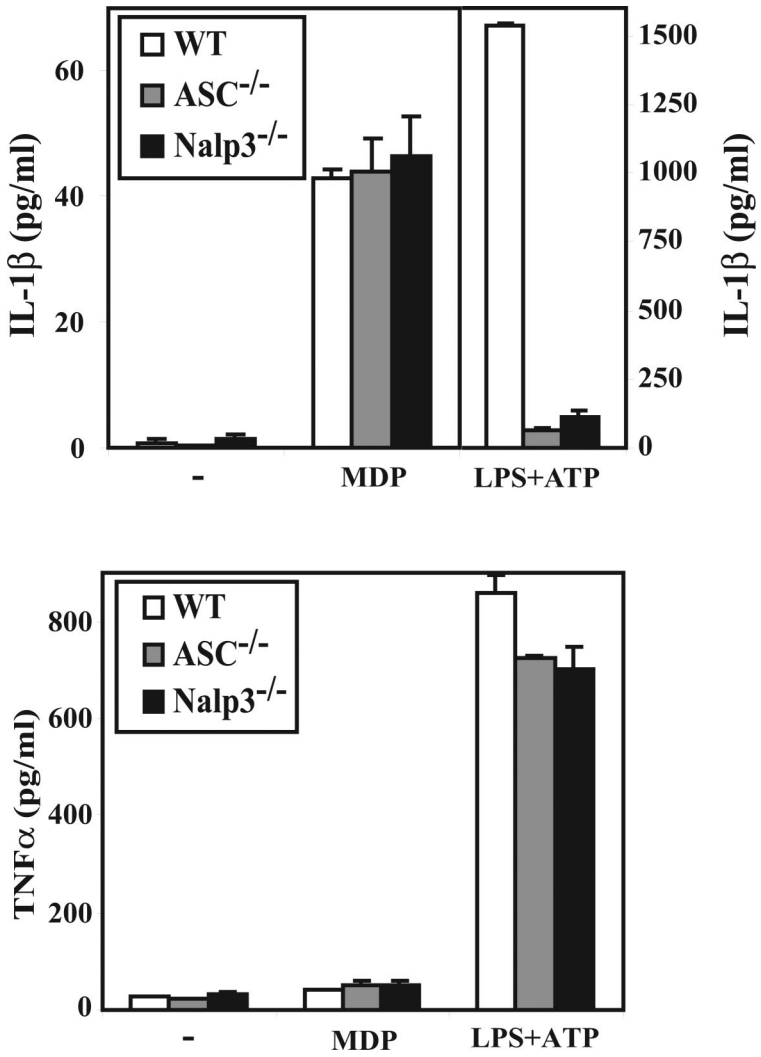

Fig. S5. ASC and NALP3 are not required for MDP-stimulated IL-1β secretion. Peritoneal macrophages from WT, *Nalp3*$^{-/-}$ and *Asc*$^{-/-}$ mice were incubated with TiO_2 microparticles without or with MDP or with LPS+ATP as indicated. After 16 h, secreted IL-1β and TNF-α were quantitated by ELISA.

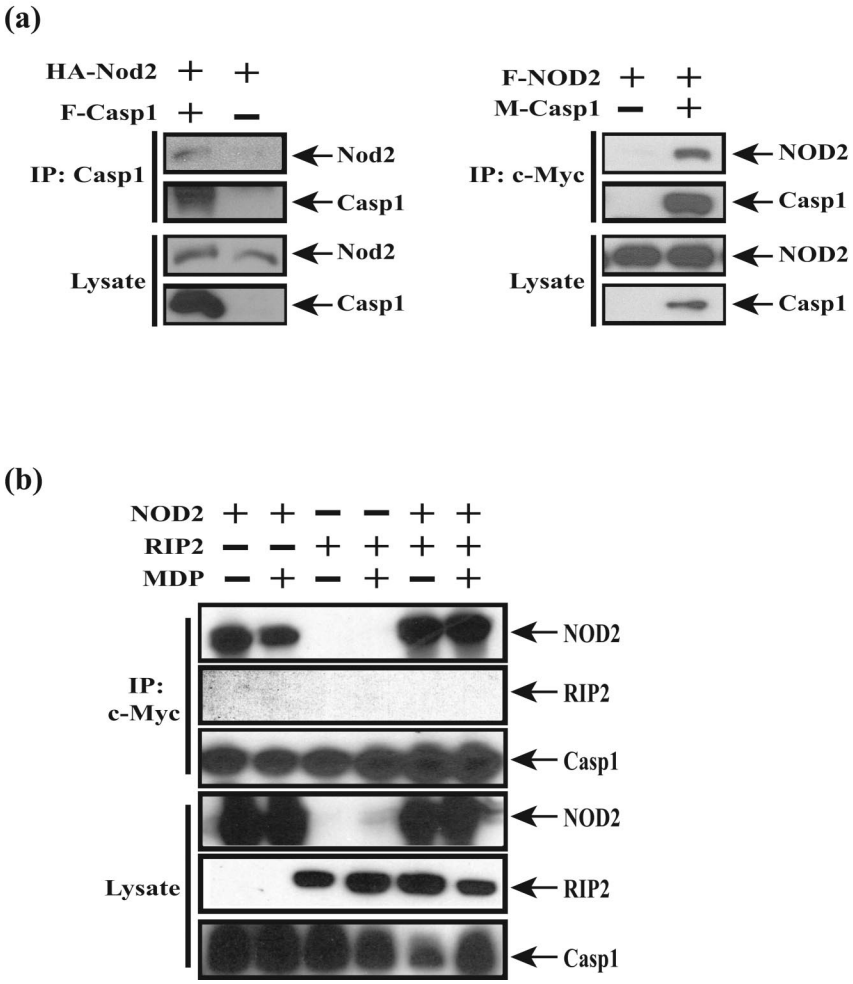

Fig. S6. NOD2, but not RIP2, interacts with caspase-1. (*A*) Mouse and human NOD2 proteins bind caspase-1. FLAG-tagged caspase-1 was co-expressed in HEK293T cells with HA-tagged mouse (left panel) or FLAG-tagged human (right panel) NOD2 constructs. Caspase-1 was immunoprecipitated and presence of associated NOD2 proteins was examined by immunoblotting. (*B*) RIP2 does not interact with caspase-1. c-Myc-tagged caspase-1 was transfected with either RIP2 or NOD2 constructs into HEK293T cells. Caspase-1 was immunoprecipitated with anti-Myc and presence of associated NOD2 or RIP2 was examined by immunoblotting of cell lysates. RIP2 was analyzed by using an anti-HA antibody from Santa Cruz.

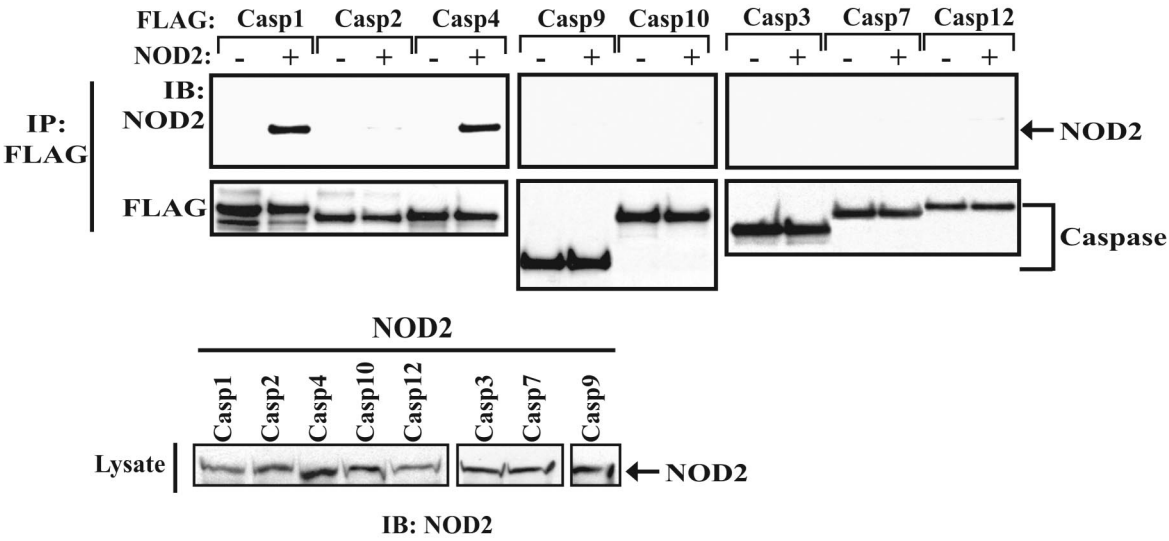

Fig. S7. NOD2 specifically interacts with caspase-1 and caspase-4, but not with other caspases. NOD2 was co-expressed in HEK293T cells with the indicated FLAG-tagged human caspase constructs. After 36 h, anti-FLAG-caspase immunoprecipitates were prepared and analyzed for presence of NOD2 and FLAG-caspases by immunoblotting. Expression of NOD2 was analyzed by immunoblotting.

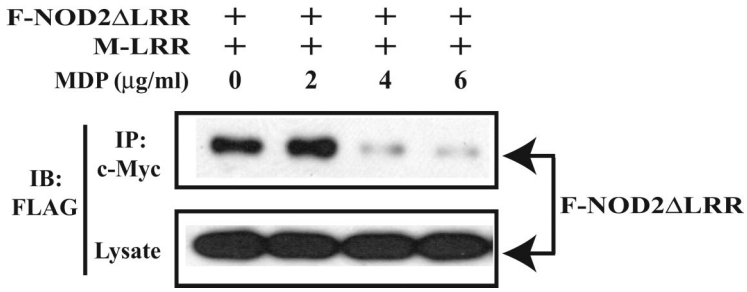

Fig. S8. MDP disrupts binding of the NOD2 LRR to the N-terminal half of the protein. HEK293T cells were transfected with the indicated human NOD2 fragments in the absence or presence of different MDP concentrations. After 36 h, the Myc-tagged LRR fragment was immunoprecipitated and coprecipitation of the FLAG-tagged NOD2ΔLRR N-terminal fragment was examined by immunoblotting.

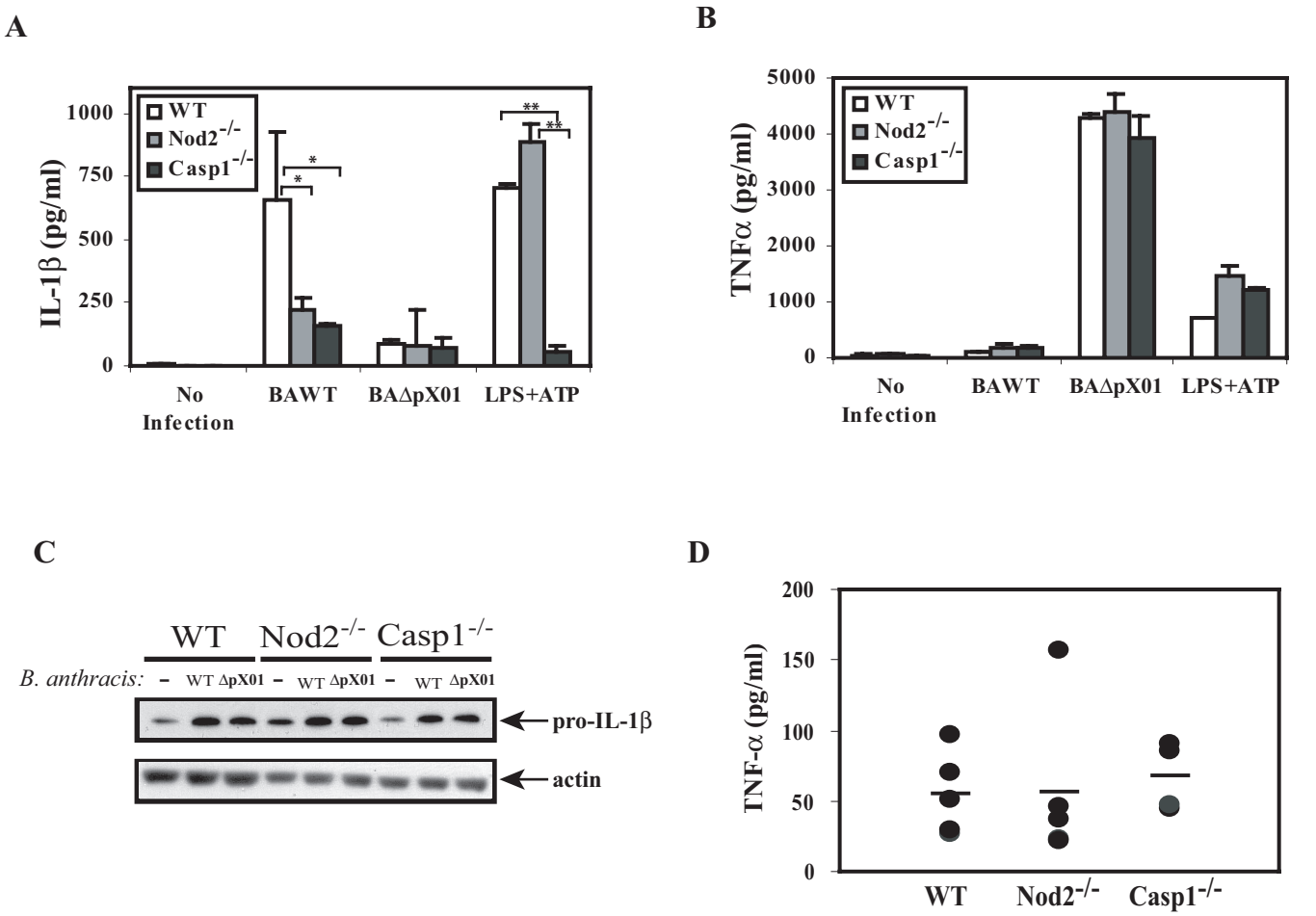

Fig. S9. IL-1β, but not TNF-α, secretion in *B. anthracis*-infected macrophages is NOD2-dependent. (*A* and *B*) IL-1β secretion by *B. anthracis*-infected macrophages. Peritoneal macrophages from WT, *Nod2*$^{-/-}$ and *caspase1*$^{-/-}$ mice were infected or not with the indicated *B. anthracis* Sterne strains (BaWT, or BaΔpX01) at moi of 2. Macrophages were also pretreated with LPS and then pulsed with ATP as a positive control. Supernatants were collected 6 h postinfection and secreted IL-1β (*A*) and TNF-α (*B*) were measured by ELISA. Significant differences, $^{**}P < 0.01$, $^{*}P < 0.05$. (*C*) NOD2 or caspase-1 is not required for induction of pro-IL-1β. Macrophages from the indicated strains were infected as (*A*) and pro-IL-1β expression was examined by immunoblotting. (*D*) TNF-α secretion in *B. anthracis* infected mice does not require caspase-1 and NOD2. Mice ($n=5$) were injected intraperitoneally with 10^7 cfu of early log phase *B. anthracis* BaWT. TNF-α in plasm was measured 17 h after infection.